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Attraction of male winterform pear psylla to female-produced volatiles and to female extracts and evidence of male–male repellency

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Abstract

Pear psylla, *Cacopsylla pyricola* (Förster) (Homoptera: Psyllidae), is a major pest of commercial pears in North America and Europe. Olfactometer trials have shown that males of both the summer and winter morphotype are attracted to female-infested host material. Additional work with the summer morphotype has shown that males are attracted to females even in the absence of the host plant, which is evidence that female *C. pyricola* produce a volatile sex attractant. Here, we describe similar results with the winterform, confirming for this morphotype that the female psylla rather than the infested host material is the source of the attractant. Male winterforms displayed attraction to odors from live females in the absence of the host plant, freshly killed females, and female whole body extracts. The female whole body extracts were at least as attractive as a comparable number of live females, suggesting that we were successful at extracting the components of the attractant with this procedure. All previous olfactometer trials with *C. pyricola* used the insect as the attractant source; the current study is the first to demonstrate that volatile chemicals isolated from the female insect were attractive to male conspecifics. Winterform males were also assayed to odors produced by conspecific males. We found that male psylla avoided volatile odors from live males, freshly killed males, or whole body extracts of males. To our knowledge, these results are the first indication that males of any member of the Psyllidae avoid odors associated with conspecific males.

Introduction

Pear psylla, *Cacopsylla pyricola* (Förster) (Homoptera: Psyllidae), is one of the most important pests of commercial pears, *Pyrus* L. (Rosaceae), in North America and Europe. Pear psylla are seasonally dimorphic, with a dark, larger, overwintering adult stage (winterform), and a smaller, lighter colored 'summerform' occurring during the growing season. In late summer and early autumn, the shortening photoperiods lead to the production of the winter morphotype (Oldfield, 1970). The winterform overwinters in reproductive diapause, characterized by lack of mating and absence of ovarian development (Krysan & Higbee, 1990). In the central Washington (USA) study area, mating begins in mid-February when temperatures begin to warm (Krysan & Higbee, 1990; Horton et al., 1998, 2007). The summer morphotype first appears in orchards beginning in early to mid-May. Females of this morphotype reach reproductive maturity within a few hours of eclosion and males become reproductive after 5 days (Burts & Fischer, 1967). There are 3–4 generations of summerforms per season in the study area.

Until very recently, the role of chemical cues in mate-locating behavior had not been investigated for Psyllidae. In 2004, Soroker and colleagues suggested that sex attraction in another pear psyllid, *Cacopsylla bidens* (Šulc), was mediated in part by female-produced volatiles (Soroker et al., 2004). Recent studies with *C. pyricola* indicated that winterform males are attracted to volatile chemicals from pear shoots infested with post-diapause female psylla (Horton & Landolt, 2007; Horton et al., 2007). Furthermore, summerform males are attracted to volatiles from summerform female-infested pear seedlings, and to females in the absence of the host plant (Horton et al., 2008). Male attraction to female-produced volatiles has very recently also been described for a psyllid other than a *Cacopsylla* species, the citrus psyllid, *Diaphorina citri* (Kuwayama) (Wenninger et al., 2008).

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Previous work with post-diapause winterform *C. pyricola* indicated that males were attracted to female-infested pear shoots (Horton & Landolt, 2007; Horton et al., 2007), but did not address the source of the attractant, that is, the female-infested host plant or the females. Studies with the summer morphotype did show that males are attracted to females even in the absence of the host plant, which suggests that the females are the source of the attraction rather than the infested foliage (Horton et al., 2008). Our first objective in the present study was to test whether such attraction occurs in the winter morphotype. We used both live and freshly killed females as the source of the attractants, as done previously in our studies with the summer morphotype (Horton et al., 2008).

Ultimate goals are to isolate and identify the chemicals responsible for attraction, requiring that we develop a method to isolate the volatile chemicals and confirm their biological activity. Thus, our second objective was to test whether chemicals collected through whole body extractions of female psylla are attractive to males in olfactometer tests. Cuticular extracts have been obtained for numerous insect species (Singer, 1998). Cuticular lipids, more specifically hydrocarbons, are known to act as semiochemicals with roles, such as sex attractants, aphrodisiacs, sex inhibitors, species and caste recognition cues, alarm pheromones, or aggregation pheromones (Howard & Blomquist, 1982, 2005; Howard, 1993). We compared whole body extracts against both live and freshly killed females to test whether the whole body extracts were as effective at attracting males as the female insect. This assay will allow us to determine whether the chemicals collected during whole body extractions contain the components of the attractant produced by females.

An additional objective was to assess the response of males to male-produced volatiles. Male-produced odors have been shown to affect behavior of conspecifics in a number of insect species across several insect orders (Zhang & Aldrich, 2003; Hillier & Vickers, 2004; Kirk & Hamilton, 2004). Our trials with summerform pear psylla suggested that males were repelled by volatiles associated with conspecific males (Horton et al., 2008). Here, we examined whether winterform males in olfactometers avoided odors from live males, freshly killed males, or whole body extracts of males.

Materials and methods

Source of insects

Winterform pear psylla were collected using a beat tray and aspirator from a commercial pear orchard located near Yakima, Yakima County (WA, USA) (46°52′N, 120°47′W) during February and March 2007 and 2008. Adults were separated by sex in the field and placed in groups of 500 on pear shoots in 10-l ventilated plastic containers. The containers and insects were kept at 5 °C until 1–3 days before the insects were to be assayed. Then they were transferred to a greenhouse environment at ~24 °C to prompt ovarian maturation in females. Ovarian maturity was determined by dissecting a subsample of 10 females on each collection date and every 2–5 days thereafter. Stages of ovarian development were scored from 0 to 7, where 0 is immature and 7 is fully mature (Krysan & Higbee, 1990). The first mature eggs are present at ovarian stage 5. The assays were not conducted until dissected females in the subsample were found to have reached an average ovarian score of 5 or higher, at which stage females become reproductive and attractive in the olfactometer (Horton et al., 2007).

Y-tube olfactometer

The response of male psylla to olfactory cues was assessed using a Y-tube olfactometer. The olfactometer, described in Horton & Landolt (2007), was constructed of a 2.5-cm diameter glass tube 27 cm in length, with two arms at 135° to one another, each 7 cm in length. Commercial air (Oxarc, Spokane, WA, USA) was metered through a charcoal filter, an air humidifier, and paired 1-l glass jars containing odor sources. A polytetrafluoroethylene hose (Cole-Parmer Instrument Company, Vernon Hills, IL, USA) 25 cm in length and 2 mm in diameter connected each jar to an arm of the Y-tube. Airflow through each arm of the olfactometer was maintained at 50 ml/min during the assays. Before each bioassay, air was passed through the whole system at 50 ml/min in each arm, including the jars containing the odor sources, for 15 min.

Whole body extracts

Extractions were performed between 12:00 and 16:30 hours (Pacific Standard Time). For each extraction, 50 psylla were transferred into a 7-ml glass vial containing 1 ml pentane for a period of 5 min, during which the glass vial was agitated by hand. The solvent was then transferred by Pasteur pipette to a clean glass vial (extract). Simultaneously with each extraction, the same procedure was used to obtain a 1-ml pentane solution having no psylla in it, to act as the control treatment. All pentane samples (both extracts and controls) were stored at 0 °C until the following day, approximately 1 h before the bioassays were conducted. After the pentane samples had warmed up at room temperature for approximately 30 min, they were concentrated down under nitrogen flow to ~200 µl. Filter paper disks (55 mm inside diameter; Whatmann No. 1, Cat. No. 1001055; Whatmann®, Maidstone, UK) were used as substrate for both extracts and solvent controls. Each olfactometer assay consisted of paired 1-l glass jars containing either the

extract or solvent control. The extracts and solvent controls were applied to filter papers with glass syringes (Hamilton Company®, Reno, NV, USA) and allowed to evaporate in a fume hood for 1 min. Each filter paper disk was then folded to prevent it from laying flat at the bottom of the jar. The disks were placed in the jars, and the jars were immediately attached to the olfactometer.

Assay methods

A replicate consisted of 10 males assayed one-at-a-time. Approximately 30 min preceding an assay, males were placed in a 50-ml holding vial. A single male was allowed to enter the olfactometer and was given 10 min to enter an arm of the Y-tube. Males that did not enter an arm within 10 min were discarded. Choice was defined by a male contacting the upwind end of an arm, at the point of insertion for the Teflon hose. For each replicate, five males were assayed, the arms of the olfactometer were rotated 180° horizontally, and the second group of five males was assayed. The olfactometer was dismantled and cleaned following each replicate of 10 males. Glassware and Teflon hoses were soaked in hot soapy water, rinsed with water, acetone, and hexane, and then baked in an oven at 150 °C for 2 h. Five sets of assays were conducted and comparisons within one set were randomized.

Effect of host plant on winterform male response to females. Three comparisons were made: (a) females and pear shoots vs. uninfested pear shoots, (b) females without shoots vs. empty jar, and (c) females and pear shoots vs. females without shoots. Females to be assayed were moved from plastic containers onto pear shoots in jars (assays 'a' and 'c') or into empty jars (assays 'b' and 'c') 2 h preceding the assay. Pear shoots were obtained from the same orchard that was the source of the psylla and trimmed at the base to 10–12 cm in length. The cut end of each shoot was placed in a 50-ml glass jar of tap water for use in the assay. In those assays for which the host plant was not present ('b' and 'c'), a glass jar (50 ml) of tap water was included in each of the 1-l jars. For the treatments that included females, we used 25 females per jar. We used three pear shoots in the treatments that included pear shoots.

Response by male winterforms to live winterform females and males. Two comparisons were conducted: (a) 50 females vs. empty jar, and (b) 50 males vs. empty jar. Two hours prior to each assay, psylla were moved into the 1-l glass jars for eventual attachment to the olfactometer. For this assay and the following ones, the number of psylla used as odor sources was increased from 25 in the previous assay to 50 to be consistent with the methods used in assaying whole body extracts (see below).

Response by male winterforms to freshly killed winterform females and males. Two comparisons were made: (a) 50 freshly killed females vs. empty jar, and (b) 50 freshly killed males vs. empty jar. The insects to be used as odor sources were killed by placing them in a –80 °C freezer for at least 30 min and up to 4 days. The freshly killed insects were removed from the freezer, transferred to the 1-l glass jars, and allowed to thaw for 15 min before the jars were attached to the olfactometer.

Response by male winterforms to whole body extracts of winterform females and males. Two comparisons were made: (a) female whole body extract vs. control, and (b) male whole body extract vs. control. Each whole body extract consisted of 50 psylla extracted in pentane for 5 min. We chose to use 50 insects for the extraction based upon preliminary trials with extracts in the olfactometer. For these bioassays, pentane samples were applied to filter papers immediately before the assays were conducted.

Comparison of male response to live females, freshly killed females, and whole body extracts of females. Three comparisons were made: (a) live females vs. freshly killed females, (b) freshly killed females vs. female whole body extract, and (c) live females vs. female whole body extract. In each treatment, 50 females were used. For the freshly killed females and the live females treatment, each jar contained a control filter paper onto which 200 µl of pentane was applied. Freshly killed insects were obtained by freezing the insects, as described above.

Data analysis

Statistical analyses were performed using SAS Version 9.1 for Windows (SAS Institute, 2002). We compared mean number of male psylla choosing one arm of the olfactometer vs. mean number choosing the other arm using paired sample t-tests in Proc TTEST, as described in Horton et al. (2007, 2008). The t-test assumes that the arithmetic differences between paired observations have a normal distribution (Zar, 1999). The normality assumption was tested using the Shapiro-Wilk statistic in Proc UNIVARIATE. When the normality assumption was not met, we used a signed-ranks test in Proc UNIVARIATE to analyze the paired differences (Zar, 1999; Horton et al., 2007, 2008). Significance values obtained with paired t-tests and signed-ranks tests yielded similar results, with the exception of two assays (1c and 5c). For those assays, results of both statistical tests are reported, with the appropriate test being reported first.

Results

Effect of host plant on winterform male response to females

All 450 males that were assayed made a choice within the 10 min cut-off time. Males chose pear shoots infested with

Table 1 Mean number of male winterform pear psylla that chose paired odor sources in the olfactometer

Experiment		Odor sources	n[1]	Number of males (mean ± SEM)	Statistical test[2]	Statistic	P-value
1. Presence of host plant	(1a)	Females on shoots	15	6.7 ± 0.3	Paired t-test	$t_{14} = 6.61$	<0.0001
		Shoots		3.3 ± 0.3			
	(1b)	Females alone	15	6.7 ± 0.3	Signed ranks	S = 60	<0.0001
		Empty jar		3.3 ± 0.3			
	(1c)	Females on shoots	15	4.3 ± 0.4	Paired t-test	$t_{14} = 1.73$	0.11
		Females alone		5.7 ± 0.4	Signed ranks	S = 60	<0.0001
2. Live psylla	(2a)	Females alone	12	7.2 ± 0.2	Signed ranks	S = 39	0.0005
		Empty jar		2.8 ± 0.2			
	(2b)	Males alone	12	2.6 ± 0.4	Paired t-test	$t_{11} = 6.75$	<0.0001
		Empty jar		7.4 ± 0.4			
3. Freshly killed psylla	(3a)	Females	12	6.1 ± 0.2	Signed ranks	S = 39	0.0005
		Empty jar		3.9 ± 0.2			
	(3b)	Males	12	4.0 ± 0.4	Paired t-test	$t_{11} = 2.35$	0.04
		Empty jar		6.0 ± 0.4			
4. Whole body extracts	(4a)	Female extract	12	6.8 ± 0.3	Signed ranks	S = 39	0.0005
		Control		3.2 ± 0.3			
	(4b)	Male extract	12	3.1 ± 0.3	Signed ranks	S = 39	0.0005
		Control		6.4 ± 0.3			
5. Female comparisons	(5a)	Live females	10	6.5 ± 0.4	Paired t-test	$t_9 = 3.5$	0.007
		Freshly killed females		3.5 ± 0.4			
	(5b)	Freshly killed females	10	3.9 ± 0.3	Signed ranks	S = 27.5	0.002
		Female extract		6.1 ± 0.3			
	(5c)	Live females	10	4.6 ± 0.3	Signed ranks	S = 27.5	0.002
		Female extract		5.4 ± 0.3	Paired t-test	$t_9 = 1.5$	0.17

[1]Each replicate consisted of 10 males assayed one-at-a-time (Horton et al., 2007, 2008).
[2]Both statistical test results are reported when conflicting significance values were obtained. For each of these assays, the appropriate statistical test is reported first.

females when that treatment was paired with uninfested pear shoots (Table 1-1a), with 66.7% of males choosing the female-infested shoots. Similar results were obtained when assaying males with odors from females in the absence of pear shoots (Table 1-1b); 67.3% of males chose the females. Males did not seem to show a preference when female-infested shoots were paired against an equivalent number of females in the absence of the host plant, with 56.7% of males choosing the females alone (Table 1-1c, P = 0.11 by paired t-test). However, results from the signed-ranks test suggest a preference for the females in the absence of the host plant (S = 60, P<0.0001).

Response by male winterforms to live winterform females and males

All 240 males that were assayed made a choice within 10 min. Males selected the jar containing the live females compared to the empty jar (Table 1-2a); 71.7% of males chose the females. When live males were paired with an empty jar, 74.2% of the males assayed chose the empty jar (Table 1-2b).

Response by male winterforms to freshly killed winterform females and males

All 240 males that were assayed made a choice within 10 min. Males chose the jar containing the freshly killed females compared to the empty jar (Table 1-3a); 60.8% of males chose the freshly killed females. When presented with the jar containing freshly killed males paired with an empty jar, 60.0% of males chose the empty jar (Table 1-3b).

Response by male winterforms to whole body extracts of winterform females and males

Of the 240 males that were assayed, 234 males made a choice within 10 min (98%). Males chose the filter paper that had been treated with the female whole body extract when paired with a solvent control filter paper (Table 1–4a); 68.3% of males chose the female whole body extract. Males selected the solvent control filter paper when paired with the filter paper on which the male whole body extract was applied (Table 1-4b); 64.2% of males chose the filter paper with the solvent control.

Comparison of male response to live females, freshly killed females, and whole body extracts of females

All 300 males made a choice within 10 min in this set of experiments. Males chose the live females (Table 1-5a) and the female whole body extract (Table 1-5b) when paired with freshly killed females; 65.0% of males chose the live females and 61.0% chose the female whole body extract. Males seemed to choose the female whole body extract when paired with the live females, with 54.0% of males choosing the whole body extract (Table 1-5c, $P = 0.002$ by signed-ranks test). However, results from the paired t-test indicate a lack of preference between live females and whole body extract ($t_9 = 1.5$, $P = 0.17$).

Discussion

The data presented here, combined with previous work (Horton & Landolt, 2007; Horton et al., 2007, 2008), strongly support the hypothesis that *C. pyricola* males are attracted to female-produced volatiles. Males of three psyllid species, *C. bidens*, *C. pyricola*, and *D. citri*, have now been shown to use volatile sex attractants to orient to conspecific females (Soroker et al., 2004; Horton & Landolt, 2007; Horton et al., 2007, 2008; Wenninger et al., 2008). Other cues, such as visual and acoustic signals, could also be involved in mate location by psyllids. For example, Krysan (1990) suggested that male *C. pyricola* use visual cues while approaching females, and showed that very little mating occurred in total darkness. Reduction in mating was also observed in total darkness with *D. citri* (Wenninger & Hall, 2007). Acoustic signaling in psyllids has been documented for a number of species, beginning with *Trioza nigricornis* (Förster) in 1950 (Ossiannilsson, 1950; Tishechkin, 1989, 2006; Percy et al., 2006). Acoustic signaling by means of wing vibrations in psyllids is thought to happen mainly through vibrations of the substrate and to be involved in mate location (Tishechkin, 1989, 2006; Percy et al., 2006). Wing vibrations have been observed in both male and female *C. pyricola*, although their importance in mating behavior or mating success in this species is unclear (Brown, 2008). Moreover, acoustic communication does not seem to be a prerequisite for successful mating (Tishechkin, 2006, 2007; Brown, 2008), suggesting that additional signals, such as olfactory cues, are involved in mate location.

Studies on olfactory signaling with *C. pyricola* have focused extensively on determining how life history characteristics and environmental conditions affect attractiveness of females to males (Horton & Landolt, 2007; Horton et al., 2007, 2008). In this study, we show that females of the winter morphotype are attractive to males, even in the absence of the host plant, confirming results obtained with the summer morphotype (Horton et al., 2008) and with two other psyllids, *C. bidens* (Soroker et al., 2004) and *D. citri* (Wenninger et al., 2008). Males of *C. bidens* showed attraction to females, and especially to females in the presence of the host plant (Soroker et al., 2004). In this study, the presence of the pear host did not enhance male response to female-produced volatiles. Male attraction to females in the absence of the host plant was further supported by assays in which freshly killed females were used as the odor source, confirming again that females are the source of the attractant (Horton et al., 2008).

To identify the female-produced volatile chemicals responsible for male attraction, we first need to develop methods for isolating these chemicals and an assay to confirm their biological activity. In the present study, we showed that the female-produced chemicals responsible for male attractiveness can be obtained by taking extracts from the body of whole insects. Olfactometer assays showed that winterform males were attracted to filter paper disks that had been treated with the female whole body extracts. These are the first data with *C. pyricola* to show that volatiles isolated from females are attractive to males even in the absence of the females. This is also the first report demonstrating the biological activity of whole body extracts in psyllids. Research is currently ongoing to identify the specific chemicals in the extract that are attractive to males.

Collectively, the results presented here confirm that *C. pyricola* winterform males are attracted by winterform female-produced volatiles from live females, freshly killed females, and female whole body extracts. Horton and colleagues suggested that using field-collected, freshly killed females could simplify handling and assay methods for collection and isolation of the chemical volatiles responsible for male attraction (Horton et al., 2008). However, direct comparisons of male attractiveness to live females, freshly killed females, and female whole body extracts indicated that freshly killed females were somewhat less attractive than either live females or whole body extracts. Of more interest, however, female whole body extracts were at least as attractive as a comparable number of live females, suggesting that whole body extractions are suitable for isolating the female-produced volatiles responsible for male attraction.

Studies with summerform *C. pyricola* suggested that males avoid male-produced volatiles (Horton et al., 2008). The data presented here indicate that males of the winter morphotype also are repelled by volatiles from males: they were repelled by volatiles from live males, freshly killed males, and whole body extracts of males. Because males were attracted to female whole body extracts and repelled by male whole body extracts in the olfactometer, the chemicals present in male and female whole body extracts are likely to be sex-specific. Cuticular lipids, and more

specifically cuticular hydrocarbons, have been shown to act as sex recognition cues in a number of other insect species (for reviews, see Howard & Blomquist, 1982, 2005; Singer, 1998).

To our knowledge, this is the first evidence of male-produced volatiles in psyllids. Male-produced pheromones occur in various insect orders, and may have any of several functions, that is, they may act as female and/or male attractants (Leal et al., 1998; Kirk & Hamilton, 2004), female aphrodisiacs (Hillier & Vickers, 2004), male repellents (Zhang & Aldrich, 2003), and inhibitors of female attractiveness (Andersson et al., 2003; Schulz et al., 2008). The present study and the study of Horton et al. (2008) are the first to report male-male repulsion in psyllids. Male repellents have been identified in the plant bug *Phytocoris difficilis* Knight, and were shown to repel other males in the field (Zhang & Aldrich, 2003). In this species, it was suggested that these male repellents could either deter other males from mating with a previously mated female or inhibit females from emitting a sex pheromone. With *C. pyricola*, the role of the volatile chemicals produced by males is still unknown. In this study, we tested male response to male-produced volatiles, leaving female response to these male-produced volatiles unaddressed. Studies are needed to investigate the response of female *C. pyricola* to male-produced volatiles.

In conclusion, this study confirms that male winterforms are attracted to volatiles emitted by females in the absence of the host plant and even in the absence of the female insect. This study also provides the first indication that males of any Psyllidae avoid odors associated with conspecific males. Future research will address the isolation and identification of the specific chemicals that are biologically active (attractant or repellent) to males.

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